Interleukin-6 as a Paracrine and Autocrine Growth Factor in Human Prostatic Carcinoma Cells in Vitro

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ABSTRACT

Interleukin (IL)-6 plays a significant role in genitourinary carcinomas. The present study was conducted to define the role of IL-6 in the growth of prostatic carcinoma and benign prostatic hyperplasia (BPH). An in vitro experiment was carried out using human prostatic carcinoma cell lines (LNCaP, which is androgen sensitive and slow growing, and DU145 and PC3, which are androgen insensitive and fast growing), and primary human epithelial and stromal cells derived from BPH. Cells were treated with recombinant human IL-6 or conditioned medium (CM) derived from the above cultured cells to identify possible paracrine and autocrine pathways. LNCaP was clearly responsive to exogenous IL-6 and to the CM derived from stromal cells, but not to the CM from LNCaP cells (P < 0.001). DU145 and PC3 were slightly stimulated to grow by exogenous IL-6 and the CM derived from both stromal and respective homologous cells (P < 0.01). In contrast, BPH-derived epithelial cells showed little or no response to IL-6. The stimulatory effect of CM on prostatic carcinoma cells was significantly reduced by the addition of anti-IL-6 antibody to the cell culture medium. Furthermore, the growth of DU145 and PC3 in serum-free medium was also inhibited by anti-IL-6 antibody (P < 0.001). All cell lines tested, except for LNCaP, secreted IL-6 into the culture medium. Results of reverse transcriptase-PCR analysis indicated that IL-6 receptor mRNA was present in all carcinoma cell lines but not in epithelial cells or stromal cells derived from BPH. These results suggest that IL-6 functions as a paracrine growth factor for LNCaP and as an autocrine growth factor for DU145 and PC3, but it has no stimulatory effect on epithelial cells derived from BPH.

INTRODUCTION

IL-6, originally identified as a T-cell-derived cytokine that induces final maturation of B cells into antibody-producing cells (1, 2), exhibits multiple biological activities that differ widely among various types of tissues and cells. IL-6 can enhance or inhibit proliferation in carcinoma cells (3–7). A variety of malignant tumors, including squamous cell carcinomas and adenocarcinomas, have been shown to contain or synthesize IL-6, and an autocrine growth stimulation has been suggested as the possible mechanism for the action of IL-6 (4, 8, 9). In genitourinary carcinoma, IL-6 also functions as an autocrine growth factor for renal cell carcinoma (4, 9).

In a previous study, we demonstrated that a continuous inflammatory stimulus strikingly accelerated methyl nitrosourea-initiated rat urinary bladder carcinogenesis (10–12), and that carcinogenesis in vivo was enhanced significantly by IL-6 (13). Moreover, we observed that IL-6 released by inflammatory cells and stromal cells functioned as a paracrine growth factor for bladder epithelial cells in vitro. Previous reports demonstrated the presence of IL-6 receptor and IL-6 in human prostatic carcinomas and BPH (14–16). Thus, IL-6 may play a role as an autocrine or paracrine growth factor. A more recent report suggested that endogenous IL-6 was a resistance factor for some chemotherapeutic agents, as well as an autocrine growth factor on DU145 and PC3 cells, which are androgen insensitive, highly aggressive prostatic carcinoma cell lines (17). It remains unclear whether the same mode of action of IL-6 applies to low-grade prostatic carcinoma or BPH. To determine its role, we conducted an in vitro experiment using primary cultures of stromal and epithelial cells derived from BPH, and cultures of prostatic carcinoma cell lines.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The three human prostatic carcinoma cell lines used were LNCaP, which is androgen sensitive and slow growing, and DU145 and PC3, which are androgen insensitive and fast growing. Primary cultures used in the present study were established from the specimens obtained by transurethral resection of benign prostatic tissues according to procedures described earlier (18). They were prostatic epithelial cells designated as BPH1 and BPH2, passage 1 and passage 2, respectively, and prostatic stromal cells designated as BPH-ST1, passage 6. Their epithelial or stromal cell identity was confirmed by immunohistochemical studies (18). LNCaP, DU145, PC3, and BPH-ST1 were grown in RPMI 1640 (Life Technologies, Gaithersburg, MD) with 10% FCS (Life Technologies). BPH1 and BPH2 were cultured in WAJC 404 medium (Irvine Scientific, Santa Ana, CA) supplemented with ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenous acid; Collaborative Research, Bedford, MA), epidermal growth factor (3 ng/ml, Life Technologies), bovine pituitary extract (30 μg/ml, Collaborative Research), prolactin (3 ng/ml, Sigma Chemical Co., St. Louis, MO), cholera toxin (10 ng/ml, Sigma), polyvinyl pyrolidone (2 mg/ml, Behring Diagnostics, La Jolla, CA), and penicillin (100 units/ml)/streptomycin (100 μg/ml). All cells were cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C. LNCaP, DU145, and PC3 are known to express IL-6 receptor (14, 15).

Cell Growth Assay. Cells were seeded on a flat-bottom 96-well plate (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) at 1 × 105 cells per well in RPMI 1640 with 10% FCS. Twenty-four h later, cultures were downshifted to serum-free medium to which recombinant human IL-6 (0–100 ng/ml, Genzyme Diagnostics, Cambridge, MA) or CM derived from tested cultures was added. In some experiments, polyclonal rabbit anti-human IL-6 antibody (Genzyme) at final concentration of 50 μg/ml was added to the above cultures in an attempt to neutralize any IL-6 that might be present in the medium. After 0–4 days in the culture, cell proliferation was assessed by adding 20 μg of the vital dye MTT (5 mg/ml; Sigma) to culture. The blue dye taken up by the cells after 4 h of incubation was dissolved in 0.04N HCl-isopropanol (100 μl/well), and its absorbance at 570 nm was read on an automated microplate reader (Bio-Tec, Winooski, VT; Ref. 19). Results of a preliminary study with the MTT assay showed that absorbance was directly proportional to the number of cells.

IL-6 ELISA. Levels of IL-6 in CM were measured by ELISA with the use of polyclonal rabbit anti-human IL-6 (Genzyme) as a solid-phase antibody and monoclonal mouse anti-human IL-6 (Genzyme) as a second antibody. The assay detected IL-6 at 10 pg/ml or higher in a linear fashion. Results were expressed as picograms per milliliter of CM.

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3 The abbreviations used are: IL, interleukin; BPH, benign prostatic hyperplasia; CM, conditioned medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-PCR; G3PDH, glyceraldehyde 3-phosphate dehydrogenase.
Isolation of Cytoplasmic RNA and RT-PCR. Cells grown in monolayers were harvested at an early phase of confluency. RNA was prepared by lysing of cells in a hypotonic buffer containing NP40 (Sigma), followed by removal of nuclei (20). Expression of human IL-6 receptor mRNA, and gp130 mRNA, an intermediary of IL-6 signaling (21), and G3PDH mRNA, a housekeeping gene, was detected by the RT-PCR method. The nucleotide bases used were 5'-ATGcTGGCCGTCGGCTGCGCGCTG-3' (nucleotides 1–24) as an upstream primer and 5'-TCTGAGCCTAAACCGTAGTCT-3' (nucleotides 748–768) as a downstream primer for human IL-6 receptor (22), 5'-ACCTATGAGATAGACCATCTAAA-3' (nucleotides 961–984) as an upstream primer and 5'-GGTrCTATAAAATATAGTATAATF-3' (nucleotides 1657–1680) as a downstream primer for human gp130 (21), and 5'-TGAAGGTCGGAGTCACGGA1TFGGT-3' (nucleotides 71–96) as an upstream primer and 5'-CATGTGGGCCATGAGGTCCACCAC-3' (nucleotides 1030–1053) as a downstream primer for G3PDH. One μg of cytoplasmic RNA was reverse transcribed by Molony murine leukemia virus reverse transcriptase (Life Technologies) at 42°C for 60 min in a 20-μl mixture with hexamer random primer (Life Technologies). Two μl of reverse-transcribed mixture was subjected to PCR in a 20-μl mixture 110 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 20 μM each dNTP (A, G, T, and C), 0.5 units of Taq polymerase (Cetus Perkin-Elmer, Norwalk, CT), and 0.25 pmol of primer; 0.001 .01 .1 1 10 100 Ref. 23]. Forty cycles of reaction at 94, 55, and 72°C for 60, 90, and 150 s, respectively, were carried out in a DNA Thermal Cycler (Cetus Perkin-Elmer). Amplified cDNA was subjected to electrophoresis in 1.5% agarose gels containing 100 ng/ml ethidium bromide. After electrophoresis, gels were viewed and photographed under an UV light illumination (Polaroid type 667 film, Polaroid Corp., Cambridge, MA). The authenticity of the PCR products was verified by diagnostic restriction digests with the following enzymes: IL-6 receptor, PstI; and gp130, KpnI (Life Technologies).
**Preparation of CM.** Cells were grown to confluency (approximately $2.5 \times 10^6$ cells/dish) in 100-mm dishes (Falcon) in the complete culture medium, washed 3 times with the serum-free medium, and cultured for an additional 48 h in 10 ml of the serum-free medium. The medium was collected and clarified by centrifugation at 5000 rpm.

**LPS treatment.** Cells were grown to confluency (approximately $5 \times 10^5$ cells/well) in a 6-well plate (Falcon) in the complete culture medium. The cells were washed 3 times with the serum-free medium and cultured for an additional 48 h in 2 ml of the serum-free medium with LPS (0–1000 ng/ml). The supernatants were collected, and IL-6 levels were measured by ELISA.

**RESULTS**

**Effect of Exogenous IL-6 on Growth of Prostatic Carcinoma Cells and BPH1 and BPH2 Cells in Monolayer Cultures.** IL-6 was added to individual wells at a final concentration of $1 \times 10^{-3}$, $1 \times 10^{-2}$, $1 \times 10^{-1}$, 1, 10, or 100 ng/ml. Forty-eight h later, the number of cells was assessed by MTT assay. LNCaP was clearly responsive to IL-6 with an accelerated growth at 10 and 100 ng/ml ($P < 0.001$) and marginally at 0.1 and 1 ng/ml ($P < 0.05$) compared with cells grown in the serum-free medium alone. DU145 and PC3 were stimulated to grow slightly by IL-6 at 10 and 100 ng/ml ($P < 0.05$). Neither BPH1 nor BPH2 cells showed any significant response to IL-6 (Fig. 1). Results of time course studies also revealed that IL-6 (10 and 100 ng/ml) strongly stimulated growth of LNCaP cells in a linear fashion during a 4-day observation period (Fig. 2A). DU145 and PC3 cells were able to grow in the serum-free medium (161 and 128% after 4-day culture) and were slightly stimulated by exogenous IL-6 (Fig. 2, B and C). No significant effect of IL-6 was observed on BPH1 and BPH2 cell growth (Fig. 2, D and E).

**Effect of CM of Various Cell Sources on Growth of Prostatic Carcinoma Cells and BPH1 and BPH2 Cells.** To determine whether LNCaP, DU145, PC3, BPH1, and BPH2 can be stimulated by soluble factors released by prostatic stromal cells, we assessed the effect of BPH-ST1 CM. The CM of prostatic carcinoma and BPH epithelial cells also were used to evaluate possible autocrine pathways. Paracrine stimulation of LNCaP cell growth by prostatic stromal cells was clearly demonstrated: BPH-ST1 CM ($\times 1$) stimulated growth of LNCaP (277% of control), as well as DU145 and PC3, but to a lesser degree (124 and 138% of control). LNCaP CM was not effective in stimulating its own growth (Fig. 3A). In contrast, CM of DU145 and PC3 weakly stimulated their own growth (125 and 154% of control; Fig. 3, B and C). BPH1 and BPH2 were stimulated weakly by CM derived from both the homologous cells and stromal cells (Fig. 3, D and E).

**Effect of Anti-human IL-6 Antibody on Cell Growth Stimulation by CM.** The specificity of the anti-IL-6 antibody was demonstrated by its complete inhibition of the mitogenic effect of recombinant IL-6. Anti-IL-6 antibody inhibited growth of LNCaP cells

![Fig. 3. Effect of CM on growth of prostatic carcinoma cells (LNCaP, (A), DU145 (B), and PC3 (C)) and BPH1 (D) and BPH2 (E) cells in monolayer culture. Cells (1.0 $\times 10^5$/well) were cultured in serum-free conditions. CM was added at a final concentration as indicated. Ninety-six h later, cells were counted. The results are expressed as relative ratios to the CM-free controls. Bars, SD of triplicate samples.](image-url)
IL-6 EFFECT ON PROSTATIC CARCINOMA CELLS

Fig. 4. Effect of anti-human IL-6 antibody on stimulation of growth by CM. Anti-human IL-6 antibody was added into medium at a final concentration of 50 μg/ml in the presence or absence of CM (×1) in an attempt to inhibit the mitogenic activity of the CM. Ninety-six h later, cells were counted. The results are expressed as relative ratios to the respective controls. A, LNCaP; B, DU145; C, PC3; D, BPH1. Bars, SD of triplicate samples.

Expression of IL-6 Receptor and of IL-6 Signal Transducer, gp130 mRNA. Expression of IL-6 receptor and an intermediary of IL-6 signaling, gp130 mRNA, was examined by the RT-PCR method. The specific PCR product for IL-6 receptor (768 bp) was observed with LNCaP, DU145, and PC3, but not with epithelial or stromal cells derived from BPH (Fig. 6, top). In all of the cell lines tested, a band specific for gp130 (720 bp) was observed (Fig. 6, middle). The lack of RT-PCR product in BPH1 and BPH2 was not due to defective primers, because the expressed 768-bp product was detected in the RNAs isolated from all of the prostatic carcinoma cell lines tested. Failure to detect IL-6 receptor was not likely due to RNA degradation because the same reverse-transcribed mixture yielded a positive prod-

Fig. 5. IL-6 production and release into CM by prostatic carcinoma cells and BPH1, BPH2, and BPH-ST1 cells. The levels of IL-6 in CM derived from a variety of cells were measured by ELISA. Bars, SD of triplicate samples.

IL-6 Production and Release into CM by Prostatic Carcinoma, BPH1, BPH2, and BPH-ST1 Cells. To determine whether IL-6 was produced by LNCaP, DU145, PC3, BPH1, BPH2, and BPH-ST1, we analyzed CM for IL-6 with ELISA. IL-6 was secreted in a large quantity by DU145, PC3, and BPH-ST1 (2819 ± 31, 3221 ± 22, and 3331 ± 208 pg/ml/48 h/3 × 10⁶ cells, respectively). BPH1 and BPH2 secreted IL-6 at lower levels than DU145, PC3, and BPH-ST1 (1124 ± 70 and 829 ± 59 pg/ml/48 h/3 × 10⁶ cells, respectively), whereas LNCaP secreted IL-6 at a marginal level (121 ± 11 pg/ml/48 h/3 × 10⁶ cells; Fig. 5).
The amount of IL-6 secreted by LPS-treated LNCaP was only marginally increased (Fig. 7).

**DISCUSSION**

Results of the present study have demonstrated the following three aspects regarding the role of IL-6 in growth regulation of benign and malignant prostatic cells. (a) Stromal and epithelial cells derived from BPH, androgen-insensitive prostatic carcinoma cell lines DU145 and PC3, produced and released IL-6 into CM far greater in quantity than BPH, androgen-sensitive prostatic carcinoma cell lines DU145 and PC3 weakly responded to exogenous IL-6 and CM from both stromal cells in a dose-dependent manner, but not by CM from LNCaP itself. DU145 and PC3 weakly responded to exogenous IL-6 and CM from both homologous cells and stromal cells. The response of these cells to IL-6 or CM was reduced significantly by the addition of anti-IL-6 antibody. Gleave et al. (27) showed that fibroblasts modulate prostatic carcinoma growth through the release of paracrine-mediated growth factors, possibly including basic fibroblast growth factor, but not epidermal growth factor, transforming growth factor-α, or transforming growth factor-β (27). Prostatic stromal cells also secrete keratinocyte growth factor and hepatocyte growth factor, both of which stimulate proliferation in epithelial cells (28-30).

Finally, treatment with LPS increased IL-6 secretion by cells, especially stromal cells. Our previous data indicate that LPS enhanced rat urinary bladder carcinogenesis in vivo and IL-6 in vitro. Prostatitis is common in men over the age of 50. Therefore, chronic urinary tract infection may have a role in stimulating the growth of prostatic carcinomas.

We conclude that acquisition of IL-6 receptor expression may be a common manifestation of prostatic carcinoma and may give it a growth advantage over nonneoplastic epithelial cells.

Our future investigation will focus on: (a) the expression of IL-6 receptor in other BPH-epithelial cells; (b) whether androgen-mediated growth of LNCaP involves IL-6; and (c) whether overexpression of IL-6 in LNCaP may induce androgen-independent phenotype and enhance its malignancy.

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